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Matrix Metalloproteinase (MMP)-9 Induced by Wnt Signaling Increases the Proliferation and Migration of Embryonic Neural Stem Cells at Low O_2 Levels*

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Recent studies have shown that various neural and embryonic stem cells cultured in 1–8% oxygen (O_2), levels lower than those typically used in cell culture (20.9%), displayed increased rates of proliferation; however, the molecular mechanisms underlying these changes are largely undefined. In this study, using rigorously controlled O_2 levels, we found that neural stem cells (NSCs) from embryonic day 15 rat cortex increased their rate of proliferation and migration in 1% O_2 relative to 20% O_2 without changes in viability. We sought to identify molecular changes in NSCs grown in 1% O_2 that might account for these increases. In 1% O_2, levels of the hypoxia-inducible transcription factor HIF-1α were transiently increased. Reduced adherence of NSCs in 1% O_2 to basement membrane-coated plates was observed, and quantitative RT-PCR analysis confirmed that the levels of mRNA for an assortment of cell adhesion and extracellular matrix molecules were altered. Most notable was a 5-fold increase in matrix metalloproteinase (MMP)-9 mRNA. Specific inhibition of MMP-9 activity, verified using a fluorescent substrate assay, prevented the increase in proliferation and migration in 1% O_2. The canonical Wnt pathway was recently shown to be activated in stem cells in low O_2 via HIF-1α. Inhibition of Wnt signaling by DKK-1 also prevented the increase in proliferation, migration, and MMP-9 expression. Thus, MMP-9 is a key molecular effector, downstream of HIF-1α and Wnt activation, responsible for increased rates of NSC proliferation and migration in 1% O_2.

A frequently overlooked variable for the *in vitro* expansion of stem/progenitor cells is the level of O_2 in cell culture (1–3). In fact, the level of O_2 (20.9% O_2) used for *in vitro* culture is hyperoxic for cells *in vivo*. The lung alveoli and bloodstream typically hold ~14% (4, 5) and 11.5% (6–8) O_2, respectively, and O_2 levels in various areas of the brain range from 0.1 to 9% (5, 7, 9–12). Recent studies have shown that the use of lower O_2 levels in culture results in increased rates of proliferation of neural stem/progenitor cells (NSCs)[2] from various embryonic or adult brain regions (12–23), virally immortalized human NSCs (23), and embryonic stem (ES) cells (3, 24, 25) and that these O_2 levels can also influence the differentiation of NSCs (9–16).

A large body of work has described hypoxia-inducible transcription factors as key components of the O_2-sensing machinery that responds to lowered O_2 in cells and organisms (5, 10, 26, 27). It has been shown that levels of HIF-1α protein can vary significantly over physiological ranges of O_2 (28). Although HIF-1α is constitutively expressed, when O_2 levels are high, the protein is hydroxylated by a family of prolyl hydroxylase enzymes, catalyzing a modification that targets HIF-1α for proteasomal degradation. At lower O_2 levels, the HIF-1α protein undergoes less hydroxylation, is stabilized, and can then translocate to the nucleus where it activates several genes that modulate the cellular response to decreased O_2 levels (29). Identification of downstream targets of HIF-1α in NSCs cultured in 1% O_2 is therefore critical for understanding the regulation of these cells *in vivo* because this level of O_2 is physiological in areas of the brain where NSCs proliferate and migrate (5, 10, 26).

Molecular targets of HIF-1α identified in NSCs (19, 30–34) include VEGF (31) and erythropoietin (32). HIF-1α stabilization by lowered O_2 was recently shown to activate Wnt/β-catenin leading to increased proliferation in cultures of ES cells and P19 cells (24). In addition, mice lacking HIF-1α had decreases in Wnt signaling and impaired neurogenesis *in vivo* (24). The Wnt pathway has also been shown to be an important effector of NSC proliferation *in vitro* (24, 35, 36). These studies strongly link low O_2 to activation of the canonical Wnt signaling pathway and effects on cell proliferation. The downstream effectors of Wnt signaling have a wide variety of transcriptional gene targets, including VEGF (37) and MMP-9 (38), but those molecules that mediate effects on NSC proliferation remain largely unknown.

In this study, we identified MMP-9 as the molecular effector of increased NSC proliferation and migration in 1% O_2. The control of MMP-9 expression in 1% O_2 was shown to be downstream of Wnt signaling. In addition, mRNA levels for several other cell adhesion and extracellular matrix proteins were stably altered in 1% O_2 suggesting changes in the overall cellular environment. These findings support the idea that O_2 is a critical variable in NSC proliferation and migration and that O_2 levels in culture should be chosen to more appropriately mimic the O_2 levels in particular cellular niches *in vivo*.

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[2] The abbreviations used are: NSC, neural stem cell; qRT-PCR, quantitative RT-PCR; ANOVA, analysis of variance; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; MMP, matrix metalloproteinase.

EXPERIMENTAL PROCEDURES

Reagents and Western Blotting—Reagents were purchased from Invitrogen unless otherwise noted. Special care was taken to prevent the degradation of HIF-1α in protein extracts. Cells in 6-well plates were washed in PBS for 5 min in the presence of the proteasome inhibitor MG132 (5 μM) to stabilize the HIF-1α protein after which time RIPA buffer (100 μl) with MG132 was added. Cells were then scraped off the plate and placed on ice for 20 min, with vortexing every 5 min. Lysed cells were centrifuged at 13,000 $\times$ *g* for 10 min to pellet the cell debris. The supernatant was transferred to a new tube and immediately used for Western blotting. Protein was loaded at 100 μg per gel lane. HIF-1α (Abcam, Cambridge, MA) primary antibody was used at 1:500. Western blots were visualized using a Qdot kit, in which biotinylated secondary antibodies (1:2000) are revealed by binding avidin-conjugated fluorescent (625 nm emission) Quantum dots. Imaging of the resultant blots was performed using a UV box (UV Products, Upland, CA) along with AlphaEaseFC software (Alpha Innotech/Cell Biosciences, Santa Clara, CA).

Primary Cell Cultures and O_2 Environment—Multipotent neural stem cells from embryonic day 15 Wistar rat cortex were isolated and cultured as described previously (15). Cells were grown on poly-L-lysine and laminin-coated surfaces in Neurobasal medium containing B27 supplement, penicillin/streptomycin, and 20 ng/ml FGF2 (Sigma) at 37 °C. Cells were previously characterized as nestin-positive, proliferative, and multipotent neural progenitors with the potential to differentiate into neurons, astrocytes, and oligodendrocytes after FGF2 removal. Moreover, over a 24-h period 99% of the cells incorporated BrdU indicating that the entire cell population is proliferative (15).

Culture vessels were placed into sealed incubators (Billups-Rothenberg, Del Mar, CA) that were perfused with various $O_2/N_2/CO_2$ mixtures (O_2 = variable as noted, CO_2 = 5%, N_2 = balance) twice a day. For cultures grown at various O_2 levels, a Biosensor plate (BD Biosciences) was used to verify the level of O_2 in the culture medium. An O_2-sensitive fluorophore quenched by O_2 is embedded in a solid substrate within each well of a 96-well plate; lowering O_2 levels in the medium increases the fluorescence. Calculations were made as suggested by BD Biosciences Technical Bulletin 443 using 20.9% ambient O_2 as a maximum value.

Cell Proliferation and MTT Assays—Cells were seeded at a density of 2000 cells/cm^2 on 96-well plates in a 200-μl volume. Cells were grown in 0.1, 1, or 20% O_2 for 6 days *in vitro*). Cell number was measured on a standard microscope by manual counting of cells using a grid with an area of 0.25 mm^2. The MTT assay was performed by addition of MTT (Sigma) to a final concentration of 0.5 mg/ml of medium. After 4 h, the medium was removed and 200 μl of DMSO (Sigma) was added to each well. After 5 min of shaking at room temperature, 150 μl of soluble material was placed into a new 96-well plate, and absorbance was read at 562 nm with a background subtraction at 660 nm.

qRT-PCR—Cells were seeded onto 10-cm plates (Corning Glass, Corning, NY) at 2000 cells/cm^2 and grown for 3 days *in vitro*. RNA was isolated using the RNeasy kit (Qiagen, Germantown, MD), and cDNA was amplified using the RT^2 first strand kit (Qiagen). An RT^2-Profiler PCR array for rat extracellular matrix and adhesion molecules (SABiosciences, Qiagen, Germantown, MD) was used to determine relative mRNA levels measured on an Applied Biosystems 7300 real time thermocycler (Carlsbad, CA). Additional qRT-PCR was performed using primers for HIF-1α (forward, aagtctagggatgcagcacgat; reverse, acatgaatgtggcctgtgcagt), VEGF (forward, aaacctcaccaaagccagcaca; reverse, aatggcgaatccagttccacga), and MMP-9 (forward, acgtgggcaaattccaaaccttcg; reverse, atccgtggtgcaggacaaatagga). Software on the SABiosciences website was used to calculate the mRNA levels.

Cell Migration—Cells were seeded onto coated glass bottom chamber slides (4-well, Lab-Tek) at a clonal density (100 cells/cm^2). Cell-to-cell distance between each cell and every other cell within the colony was estimated as the distance between the centers of nuclei revealed by Hoechst 33342 staining. Measurements were taken from the 4- to 32-cell stage (4–6 days *in vitro*) and quantified using Slidebook 5 software.

Zymography and MMP-9 Activity Assay—For both assays, cells were seeded onto 6-well plates at ~150,000 cells/well in 2 ml of Neurobasal media supplemented as described above. After 1 day, cells were treated with 5 nM MMP-9 inhibitor or 300 ng/ml DKK-1, and aliquots of media were collected after 2 more days for both assays. Zymography was performed by loading 10 μl of culture supernatant (in nondenaturing sample buffer) per well onto a Bio-Rad 1% gelatin gel. Following electrophoresis, the gel was incubated in 2.7% Triton X-100 (Sigma) in water with shaking for 30 min. The gel was briefly rinsed with water after which time developing (digestion) buffer (50 mM Tris base, 40 mM HCl, 200 mM NaCl, 5 mM $CaCl_2$, 0.2% Brij 35) was added. Digestion of gelatin was allowed to occur for 18–20 h at 37 °C at which time the gel was rinsed and stained with 0.5% Coomassie Blue, 30% methanol, 10% acetic acid for 1 h. The gel was rinsed and de-stained with 5% methanol, 30% acetic acid for 1 h. White bands on a blue background were then digitally captured. The MMP-9 enzyme activity assay (Fluorokine E, R&D Systems, Minneapolis, MN) was performed exactly as recommended in the instructions for the kit.

Statistical Analysis—Statistics were performed with a paired, type II, two-tailed *t* test (MS Excel, Microsoft, Redmond, WA) and repeated with one-way ANOVA (SPSS 16, IBM, Somers, NY) along with a post hoc Fisher's least significant difference test (SPSS 16). *, $p < 0.05$; **, $p < 0.01$.

RESULTS

Measurement of O_2 Levels in the Medium of NSC Cultures—Lowering the O_2 level in cell culture has been shown to increase the proliferation rate of NSCs, but the final levels of O_2 and the kinetics of equilibration of the gaseous atmosphere and the culture medium were not carefully controlled or reported (12–23). Tanks with premeasured amounts of O_2 at 0.1, 1, or 20% were used to perfuse sealed incubator chambers. Full equilibration of O_2 in the culture medium to the levels in the perfused atmosphere occurred within 6–8 h (Fig. 1) similar to the time course found in cultures of placental trophoblast cells (39). During the course of the experiments, cells were exposed to atmospheric

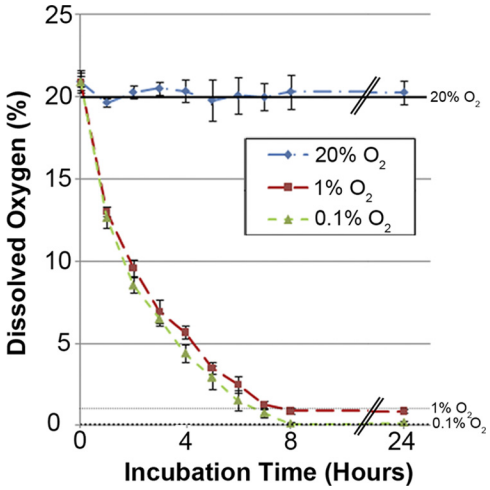

FIGURE 1. **Levels of O_2 equilibrate over time after perfusion of sealed incubator chambers.** Gas mixtures containing 20, 1, or 0.1% O_2 were perfused into sealed incubator chambers, and the amount of O_2 dissolved in the medium was determined over a 24-h period. The experiment was repeated two times with eight samples/run. *Error bars* represent the standard deviation.

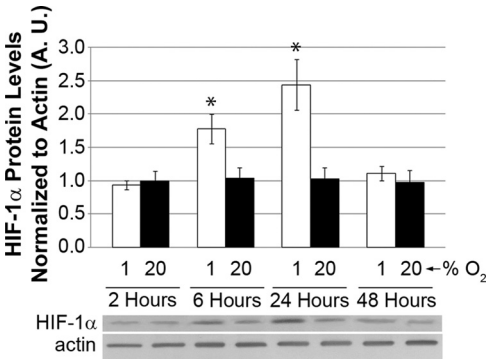

FIGURE 2. **HIF-1α protein is transiently stabilized in NSCs in 1% O_2.** HIF-1α protein levels were measured over a 48-h period in NSCs grown in 20% (*black bars*) or 1% O_2 (*white bars*); levels are expressed relative to those at 2 h in 20% O_2 and are normalized to actin (representative Western blots are shown *below* the graph). The experiment was repeated three times ($n = 3$). *, $p < 0.05$, by one-way ANOVA with a post hoc Fisher's least significant difference test. *Error bars* represent the standard deviation. *A.U.*, arbitrary units.

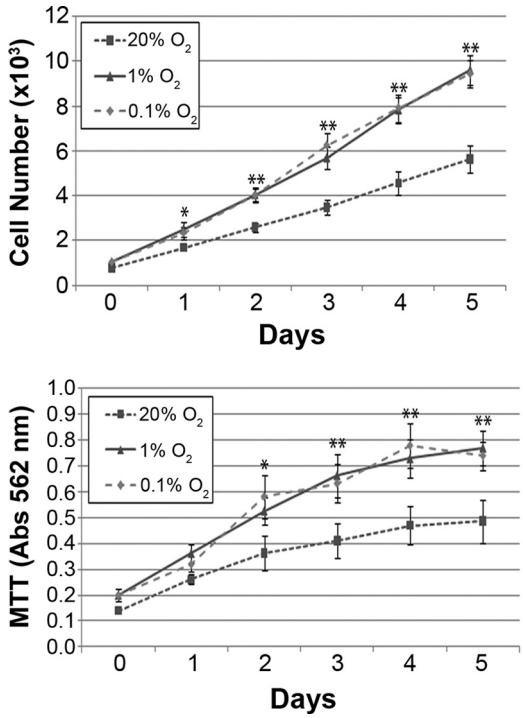

FIGURE 3. **Proliferation rate of NSCs in various O_2 levels.** *Top* proliferation was measured by counting the number of cells in a 0.25-mm^2 area. *Bottom* MTT assay was performed over the same time period, and measurements of absorbance (*Abs*) at 562 nm were corrected using a background subtraction of values at 660 nm. Both experiments were repeated four times with 4–6 samples/run ($n = 18$). *, $p < 0.05$; **, $p < 0.01$ by one-way ANOVA with a post hoc Fisher's least significant difference test. *Error bars* represent the standard deviation.

O_2 for brief periods for medium changes and microscopic examination. Additional experiments (data not shown) showed that the equilibrated O_2 levels did not increase significantly over the first half-hour of exposure to air. To maintain constant O_2 levels, medium was pre-equilibrated by exposure to gas mixtures for 6–8 h, and exposure of cultures to ambient air was limited to 30 min during experiments thus maintaining a constant level of O_2 throughout the experiment.

Transient Elevation of HIF-1α Protein Levels in NSCs in 1% O_2—The stabilization of HIF-1α protein is a consistent indicator of lowered O_2 levels (5, 10, 26, 27). HIF-1α protein levels were measured by Western blotting and normalized to the level of actin in cultures 2, 6, 24, or 48 h after cells were placed into either 1 or 20% O_2 atmospheres (Fig. 2). HIF-1α protein levels were indistinguishable after 2 h in 1% *versus* 20% O_2 levels, were elevated 1.7-fold by 6 h and 2.5-fold by 24 h in 1% O_2, and returned to base-line levels at 48 h. Levels of HIF-1α protein in NSCs in 20% O_2 remained constant over this period. Levels of HIF-1α mRNA were unchanged over 48 h and VEGF mRNA was elevated 2-fold at this time (data not shown) as expected from previous studies in many cell types (40–42).

Proliferation of NSCs Is Increased in 1 or 0.1% O_2 Compared with 20% O_2—The proliferation rate of NSCs was measured by either manual counting (Fig. 3*A*) or the MTT assay (Fig. 3*B*) (43–46). Relative to cells grown in 20% O_2, NSCs grown in 1 or 0.1% O_2 exhibited a 40% increase in proliferation rate. The MTT assay is based on the reduction of a substrate to a colored product; thus, the finding that the results of this assay and cell counting are similar (which is not always the case (47–50)) indicates that the overall redox capabilities of NSCs at each O_2 level are not substantially different. Cell viability measured by trypan blue exclusion was also unaffected (95–99% viability) by culture in the various O_2 concentrations (data not shown). These findings are consistent with studies in the literature (12, 14, 16–23) and with our previous data (15) that NSC proliferation is increased when O_2 levels in the culture atmosphere are lowered. Given that our entire cell population is proliferative, as assessed by BrdU incorporation over 24 h (15), these results suggest that cell cycle kinetics are altered in lowered O_2 environments, a finding that will be explored in detail in future studies.

Cell Migration Is Increased in NSC Clonal Cultures in 1% O_2—NSCs were grown as clonal colonies and monitored from the single cell to the 32-cell stage to determine whether lower O_2 levels influenced cell migration. Two types of cell migration and colony formation were observed as follows one in which cells migrated to form colonies of a symmetric round area (Fig. 4, *A*

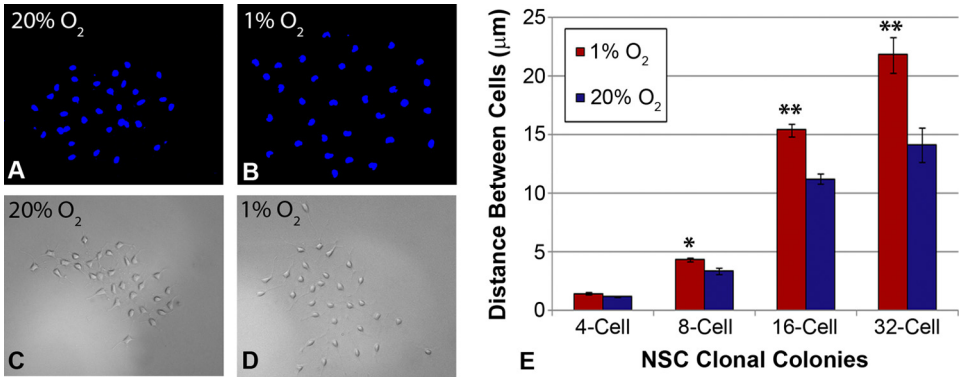

FIGURE 4. **Cell migration in NSC clonal colonies in 20 or 1% O_2.** Clonal NSC colonies were examined from the 4- to 32-cell stage. Cell-to-cell distance between each pairwise combination of cells was measured as described under "Experimental Procedures." Representative 32-cell stage colonies are shown (*A* and *B*, Hoechst-stained; *C* and *D*, bright field). Ten to 16 colonies per condition were evaluated (*E*). *, $p < 0.05$; **, $p < 0.01$ by one-way ANOVA with a post hoc Fisher's least significant difference test. *Error bars* represent the standard deviation.

TABLE 1

qRT-PCR analysis of extracellular matrix and adhesion molecules

The mRNA levels for extracellular matrix and adhesion molecules in NSCs in either 20 or 1% O_2 were measured using an SABiosciences qRT-PCR assay as described under "Experimental Procedures." Significant changes that were over 1.75-fold are included ($n = 3$). Significance was determined by one-way ANOVA with a post hoc Fisher's least significant difference test.

Symbol	cDNA target	20% O_2 cycle no.	1% O_2 cycle no.	1 *vs.* 20%	*p* value
MMP-9	Matrix metallopeptidase 9	27.00	24.59	5.03	0.009
MMP-12	Matrix metallopeptidase 12	34.66	33.25	2.51	0.003
MMP-3	Matrix metallopeptidase 3	30.29	28.87	2.54	0.045
Thbs2	Thrombospondin 2	24.99	23.99	1.89	0.012
Ldha	Lactate dehydrogenase A	19.93	18.99	1.81	0.001
Col6a1	Collagen, type VI, a1	22.61	21.70	1.77	0.006
Timp2	TIMP MMP inhibitor 2	20.42	21.22	−1.84	0.049
Ctnna2	Catenin a2	24.59	25.71	−2.29	0.019
Cdh4	Cadherin 4	24.92	26.06	−2.33	0.032
Thbs1	Thrombospondin 1	19.25	20.65	−2.79	0.024
Cdh1	Cadherin 1	31.95	33.74	−3.66	0.048

and *B*; shown stained with Hoechst dye to reveal nuclei, representing ~70% of colonies) or those in which cells migrated out into an asymmetric shape (Fig. 4, *C* and *D*; shown in bright field imaging, representing ~30% of colonies). The asymmetric colonies likely reflect an uneven substrate coating onto the glass surface. The extent of migration over 1–5 days was estimated by measuring the cell-to-cell distances within colonies using the center of a Hoechst-stained nucleus as the point of reference for each cell. The distances between cells were measured from each cell to every other cell pairwise in the same colony. Migration was measured in 4-, 8-, 16-. and 32-cell colonies (32-cell colonies are represented in Fig. 4, *A–D*). Cell migration increased over time in both conditions; however, by the 8-, 16-, and 32-cell stages, differences in the extent of migration were apparent between NSCs grown in the two O_2 levels. Cells in 1% O_2 migrated 40–50% more by the 16- and 32-cell stages than those in 20% O_2 (Fig. 4*E*).

Levels of mRNA for Cell Adhesion and Extracellular Matrix Molecules Are Altered in NSCs in 1% O_2—In addition to the increased cell migration in 1% O_2, we observed a decrease in NSC adhesion in 1% O_2 to the poly-L-lysine and laminin-coated plates, suggesting changes in cell and matrix adhesion. The mRNA levels for selected cell adhesion and extracellular matrix proteins were therefore measured using a rat extracellular matrix and adhesion molecules qRT-PCR array (see under "Experimental Procedures"). Using a 1.75-fold change as a cut-off, we found six mRNAs (of 84 genes on the array) that were increased in 1% as compared with 20% O_2 and five mRNAs that were decreased in 1% as compared with 20% O_2 (Table 1). The genes that were up-regulated in NSCs in 1% O_2 included the extracellular matrix proteins thrombospondin (TSP)-2 and collagen VIα1, the extracellular matrix-modifying enzymes matrix metalloproteinases (MMPs) MMP-3, -9, and -12, as well as lactate dehydrogenase. The up-regulation of lactate dehydrogenase A (a housekeeping gene in the array) is a likely indicator of increased glycolysis known to occur in the lower O_2 environment (51, 52). Down-regulated genes included the extracellular matrix protein TSP-1, two cadherins (E or 1 and R or 4), α2-catenin, an intracellular protein involved in cadherin signaling, and TIMP-2, a tissue inhibitor of MMPs.

All of these mRNA changes are intriguing candidates for further analysis; however, the largest increase was in MMP-9 expression, which showed a 5-fold increase in its mRNA (Table 1 and Fig. 7*A*) and an ~2.5-fold increase in protein levels measured by zymography (Fig. 7*B*) or by using a quantitative MMP-9 assay kit (Fig. 7*D*). MMP-9 influences proliferation and migration in a number of cell types (53–56), and a specific small molecule inhibitor is available (57) (the specificity of which is 20-fold greater for MMP-9 than for MMP-13 and 200-fold greater than for MMP-1). We therefore focused on the role of MMP-9 in proliferation and migration of NSCs.

Increase in Proliferation and Migration of NSCs in 1% O_2 Is Dependent upon MMP-9 Activity—NSCs were seeded and grown for 1 day at which time MMP-9 Inhibitor I was added at

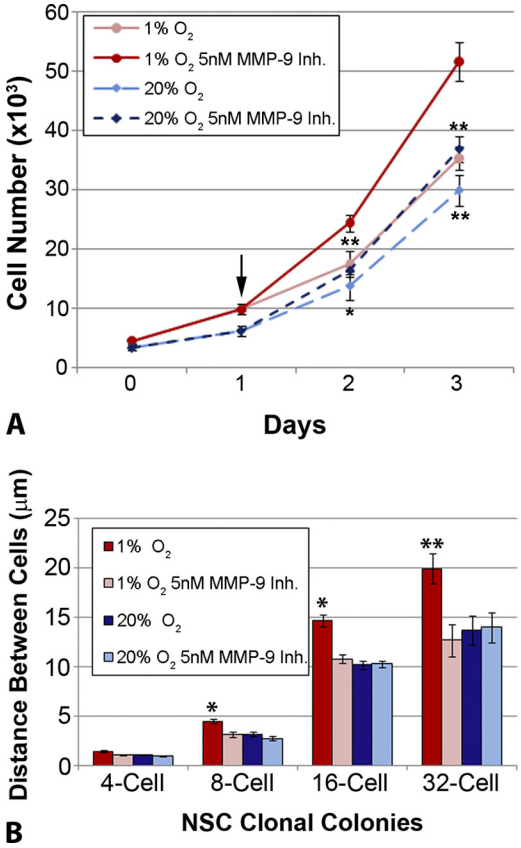

FIGURE 5. **Effect of MMP-9 inhibition on NSC proliferation and migration.** *A,* increased proliferation rate of NSCs in 1% O_2 is prevented in the presence of MMP-9 Inhibitor I. The MMP-9 inhibitor was used at 5 nM, the half-maximal concentration for MMP-9 proteolytic inhibition *in vitro* (57) and a concentration that abolished MMP-9 activity in the NSC culture medium measured using a quantitative fluorescent-substrate assay (see Fig. 7 for details) (expressed as active MMP-9 (ng)/10^5 cells: 20% control, 1.27 ± 0.17; *20%/MMP-9 Inh.*, **, 0.07 ± 0.06; 1% control, 2.28 ± 0.23; *1%/MMP-9 Inh.*, **, 0.08 ± 0.07). *B,* clonal NSC colonies were grown to the 32-cell stage in 20 or 1% O_2 ± MMP-9 Inhibitor I (5 nM). Cell-to-cell distance was measured as described under "Experimental Procedures." The experiments in *A* were repeated two times with 4–6 samples/run ($n = 10$), and the experiment in *B* was performed with 10–16 colonies per treatment ($n = 10$–16). *, $p < 0.05$; **, $p < 0.01$ by one-way ANOVA with a post hoc Fisher's least significant difference test. *Error bars* represent the standard deviation.

5 nM, and the IC_{50} value reported for inhibition of active MMP-9 was measured *in vitro* in a spectrophotometric assay (57). To confirm the efficacy of the drug in the NSC cultures, the activity of MMP-9 in the NSC culture supernatant was measured in the presence or absence of the inhibitor. MMP-9 activity was inhibited by ~94% in NSCs grown in 20% O_2 and by 96% in NSCs grown in 1% O_2 (for details, see Fig. 5 legend).

Although MMP-9 Inhibitor I had no effect on the proliferation of NSCs in 20% O_2, it completely reversed the increased rate of proliferation seen in NSCs grown in 1% O_2 (Fig. 5*A*). The doubling times of NSCs under these conditions (Table 2) were calculated from the data in Fig. 5*A* using the values from day 2 to 3 after inhibitor treatment. This interval was chosen to avoid the transition period in cell proliferation immediately following treatment of the unsynchronized cell population at day 1. However from day 1 to 2 of treatment, although the slopes appear to be slightly different during this transition period, the calculated values are not statistically different.

TABLE 2
Doubling time of proliferating NSCs

The doubling time of NSCs in 1% O_2 was decreased compared with cells in 20% O_2 or cells in either O_2 condition treated with MMP-9 inhibitor I or DKK-1. Results were obtained from days 2 to 3 to avoid the transition period immediately following treatment with either inhibitor (for Fig. 5 data, $n = 10$, and Fig. 6 data, $n = 8$). Significance was determined by one-way ANOVA with a post hoc Fisher's least significant difference test.

	Days
Fig. 5	
20% O_2	1.14 ± 0.08
20% O_2 5 nM MMP-9 inhibitor	1.16 ± 0.07
1% O_2	0.90 ± 0.11[a]
1% O_2 5 nM MMP-9 inhibitor	1.17 ± 0.13
Fig 6	
20% O_2	1.21 ± 0.15
20% O_2 300 ng/ml DKK-1	1.29 ± 0.14
1% O_2	0.91 ± 0.12[a]
1% O_2 300 ng/ml DKK-1	1.27 ± 0.14

[a] $p < 0.05$ by one-way univariate ANOVA.

The doubling time of NSCs in 20% O_2 was constant with or without MMP-9 Inhibitor I. This finding indicates that in high O_2 MMP-9 activity does not strongly influence NSC proliferation. The doubling time of NSCs in 1% O_2 was ~22% shorter than that in 20% O_2 and was restored to 20% O_2 levels when MMP-9 activity was inhibited. The increase in MMP-9 (see Fig. 7 for quantification) induced by 1% O_2 thus appears to alter cell cycle kinetics, a point that deserves a more critical analysis in future studies.

In cell migration assays, the presence of MMP-9 Inhibitor I at 5 nM prevented the increased migration of NSCs in 1% O_2 (Fig. 5*B*). To our knowledge, these results are the first evidence directly linking MMP-9 activity to increased NSC proliferation and migration in low O_2 levels.

Inhibition of Wnt/β-Catenin Signaling Prevents the Increased Proliferation and Migration in 1% O_2—MMP-9 has been shown in human T-lymphocytes to be a transcriptional target of the Wnt signaling pathway (38). A recent study showed that O_2 regulates stem cells through canonical Wnt/β-catenin signaling downstream of HIF-1α stabilization (24). We therefore determined whether perturbation of Wnt signaling influenced the proliferation, migration, and expression of MMP-9 in NSCs.

DKK-1, a specific inhibitor of the canonical Wnt/β-catenin signaling pathway (58), was used to determine whether this pathway plays a role in the increased proliferation and migration of NSCs in 1% O_2. Treatment with DKK-1 (300 ng/ml) reversed the increase in proliferation in 1% O_2 to the level of that in 20% O_2 (Fig. 6*A*). DKK-1 had no effect on the proliferation of NSCs in 20% O_2. The doubling time in 1% O_2 was ~28% shorter than in 20% O_2 or in the presence of DKK-1 at either O_2 concentration (Table 2).

In the presence of DKK-1, the increase in migration of NSCs in 1% O_2 was also reversed (Fig. 6*B*). Both of these findings, when taken together with observations in the literature (24), suggest that Wnt signaling controls MMP-9 expression in NSCs.

Inhibition of MMP-9 or Wnt Signaling Prevents the Increased Expression of MMP-9 in NSCs in 1% O_2—MMP-9 mRNA and protein levels were measured in control NSCs and NSCs treated with MMP-9 Inhibitor I or DKK-1. The 5-fold increase

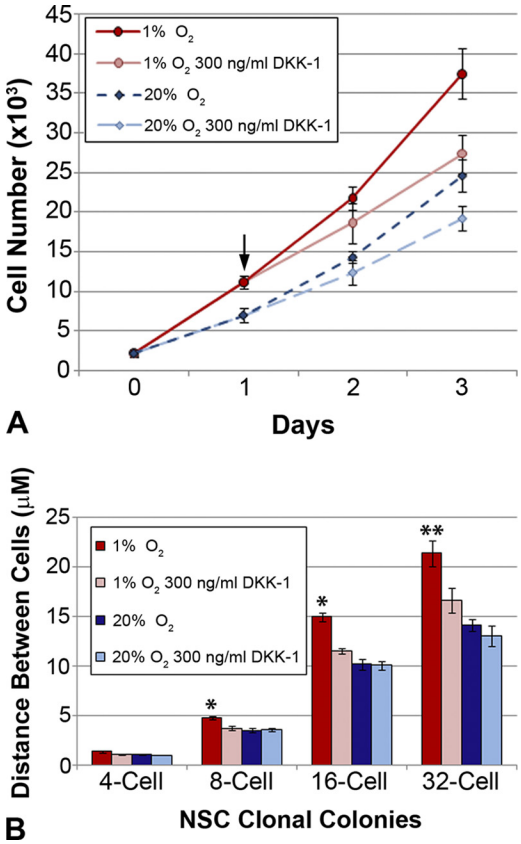

FIGURE 6. **Inhibition of Wnt signaling prevents the increase in NSC proliferation and migration in 1% O_2.** *A,* increased proliferation rate of NSCs in 1% O_2 (measured by cell counting) is prevented in the presence of the Wnt pathway inhibitor DKK-1 (300 ng/ml). *B,* clonal NSC colonies were grown to the 32-cell stage in 20 or 1% O_2 ± DKK-1 (300 ng/ml). Cell-to-cell distance was measured as described under "Experimental Procedures." The experiments in *A* were performed once with eight samples/run ($n = 8$), and the experiment in *B* was performed with 10–16 colonies per treatment ($n = 10–16$). *, $p < 0.05$; **, $p < 0.01$ by one-way ANOVA with a post hoc Fisher's least significant difference test. *Error bars* represent the standard deviation.

in MMP-9 mRNA levels (normalized to 18 S rRNA levels) in NSCs grown in 1% O_2 (Table 1) was confirmed by qRT-PCR analysis (Fig. 7*A*). MMP-9 Inhibitor I and DKK-1 had no effect on MMP-9 mRNA expression levels in 20% O_2 (Fig. 7*A*). The increase in MMP-9 mRNA levels induced in 1% O_2 was prevented by treatment with either MMP-9 Inhibitor I or DKK-1 (Fig. 7*A*). The effect of MMP-9 Inhibitor I on MMP-9 mRNA levels suggests that MMP-9 expression and/or activity influences the expression of MMP-9 mRNA in 1% O_2. Similar patterns of change in the expression of MMP-9 protein were observed by zymography (Fig. 7*B*) or by measuring total MMP-9 protein levels using a fluorescent substrate assay (Fig. 7*D*). Results using this same assay to measure enzymatically active MMP-9 showed that in each case active MMP-9 was approximately half of the total (Fig. 7*C*). It should be noted here that for the MMP-9 Inhibitor I-treated cells, MMP-9 activity was measured after the removal of the inhibitor thus revealing that neither the total MMP-9 protein nor its active fraction was irreversibly affected by the presence of the inhibitor.

Taken together, these results suggest that MMP-9 is induced in 1% O_2 by stimulation of Wnt signaling, and the increased levels of MMP-9 are the direct effector of increased NSC proliferation and migration.

DISCUSSION

In this study, we identified MMP-9 as a key molecular effector of increased NSC proliferation and migration in lowered O_2. We also demonstrated that the O_2-dependent induction of MMP-9 occurs via canonical Wnt/β-catenin signaling. In addition, the expression of several other genes involved in cell-cell and cell-extracellular matrix adhesion and matrix remodeling was stably altered in the presence of lowered O_2. The level of O_2 in culture is therefore a critical variable that should be controlled to increase the ability of cell culture to reflect the *in vivo* environment. The use of physiological O_2 environments for stem cell culture that more closely mimics the *in vivo* environment may increase the likelihood of successful use of stem cells in diagnostic and therapeutic applications.

Although our previous studies and those of others had shown that proliferation of various types of NSCs in culture is enhanced when O_2 levels are lower than the O_2 levels of ambient air typically used in cell culture (12, 14–23), and recent studies identified Wnt signaling to be activated in low O_2 levels, this study provides the first link to the activity of a molecular target, MMP-9, that is functionally responsible for the observed increases in cell proliferation. Moreover, the controlled O_2 levels in these experiments are comparable with those of the neurogenic stem cell niche in the developing and adult brain (5, 7, 10–12). Therefore, these physiological O_2 levels are not hypoxic for NSCs. The stable alterations in gene expression observed in NSCs chronically exposed to 1% O_2 are thus likely better to reflect the state of NSCs *in vivo*.

An increase in the levels of HIF-1α protein is a hallmark of the response of cells and organisms to lower levels of O_2 (5, 10, 26, 27). A transient increase in HIF-1α levels was observed in NSCs in 1% O_2, but stable changes in the expression of other genes also occurred under this condition. The mRNAs up-regulated in NSCs in 1% O_2 encode proteins that are involved in matrix remodeling and cell migration, including MMP-3, -9, and -12, as well as TSP-2 and collagen VIα1. The down-regulation of Timp2 mRNA, an endogenous inhibitor of MMPs, might also contribute to alterations in matrix remodeling.

The mRNAs found to be down-regulated include those that encode proteins that could otherwise enhance cell adhesion and signaling. For instance, altered expression of cadherins can alter cell-cell interactions and thereby release β-catenin, making it available to participate in Wnt signaling complexes (59). TSP-1 and -2 are postulated to play roles in migration and neurogenesis (60–62), and TSP-2 (but not TSP-1) enhances Notch signaling, a key pathway in neural fate determination (62). In addition, the increase in lactate dehydrogenase suggests a shift toward more glycolytic metabolism in NSCs in 1% O_2, as seen in a number of cell types in response to low O_2 (63, 64). Changes in these mRNAs indicate that a major influence of O_2 is to alter cell-cell and cell-matrix adhesion. Any or all of these cell adhesion modifications are likely to influence the proliferation and migration of NSCs.

The greatest change in mRNA levels was a 5-fold increase of MMP-9 expression in 1% O_2; MMP-9 protein was also elevated ~2.5-fold. The increased rates of NSC proliferation and migration in 1% O_2 were reversed in the presence of a specific inhib-

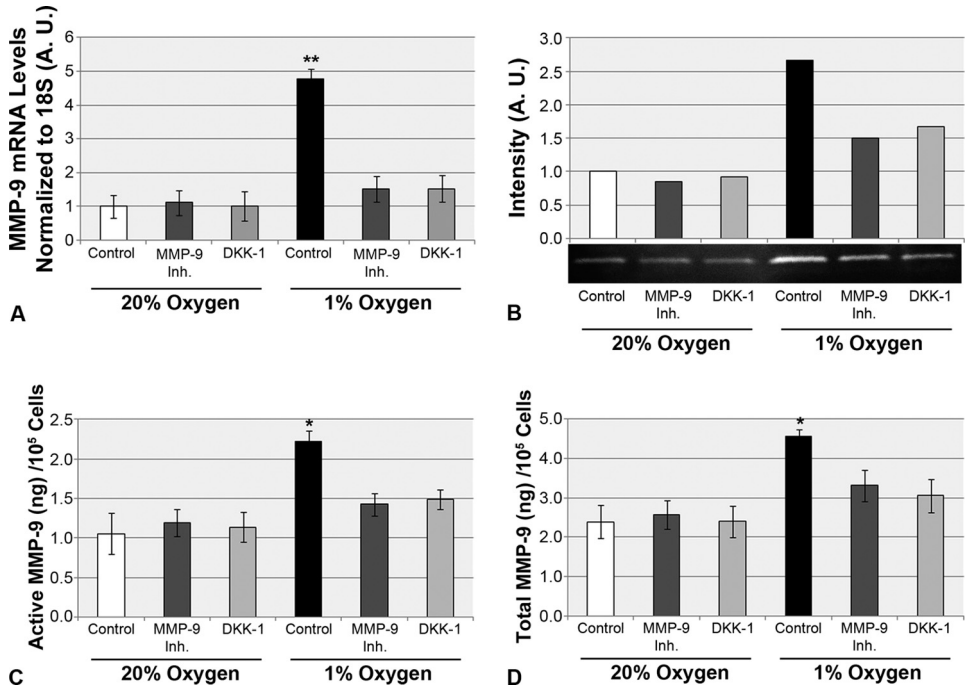

FIGURE 7. **MMP-9 expression levels in NSCs with or without inhibition of MMP-9 activity or Wnt signaling.** *A,* increased mRNA expression of MMP-9 (normalized to the levels of 18S rRNA) in 1% O_2 is decreased in the presence of MMP-9 Inhibitor I (5 nM) or DKK-1 (300 ng/ml). *B–D,* influence of inhibitors compared with untreated control cells on protein levels following 2 days of treatment with either MMP-9 Inhibitor I (5 nM) or DKK-1 (300 ng/ml) follows the same pattern as mRNA expression whether measured by zymography (*B*), active enzyme levels secreted into the medium MMP-9 (*C*), or total secreted MMP-9 (including active MMP-9 and inactive pro-MMP-9) (*D*). The experiments in *A* were performed twice with 3–4 samples/run ($n = 4–7$); the experiment in *B* was performed once (as shown), and the experiment in *C* and *D* was performed once on two sets of samples run in triplicate ($n = 6$). *, $p < 0.05$; **, $p < 0.01$ by one-way ANOVA with a post hoc Fisher's least significant difference test. *Error bars* represent the standard deviation. *A.U.*, arbitrary units.

itor of MMP-9. It is notable that inhibition of MMP-9 did not affect proliferation or migration in NSCs in 20% O_2. This finding suggests that it is a threshold of MMP-9 expression/activity that is critical for altered cell migration and proliferation. Although to our knowledge this is the first time that MMP-9 activity has been linked to NSC proliferation, MMP-9 has been shown to influence NSC migration (65–67) and differentiation (67) *in vitro* and to play a role in the response of NSCs *in vivo* after injury (67) or stroke/hypoxia (65, 66, 68). Moreover, MMP-9 is found in a number of areas of the brain, including neurogenic niches (69), and thus is likely to influence stem cell proliferation and migration *in vivo*. It will clearly be of interest to examine NSC proliferation and neurogenesis in MMP-9 null mice.

The regulation and function of MMP-9 itself are controlled at multiple levels, including expression, secretion, activation, and localization (70). With regard to expression levels, we found that inhibition of MMP-9 activity and inhibition of the canonical Wnt signaling pathway in NSCs decreased the levels of MMP-9 mRNA and protein induced in 1% O_2, but they do not affect the endogenous levels in 20% O_2. Thus, the regulation of MMP-9 expression by lowered O_2 may occur through a different pathway than the one that controls endogenous expression in 20% O_2. The mechanisms by which MMP-9 controls its own synthesis and the involvement of the Wnt pathway in MMP-9 regulation are of great interest given the wide ranging role of MMPs in the behavior of normal and cancer cells (70, 71) and in the development of the nervous system (68, 72, 73).

The mechanisms by which MMP-9 influences cell proliferation, migration, and cell cycle kinetics have not been determined. Two possibilities, not mutually exclusive and likely related, are suggested by our findings and studies in other cell types. The first mechanism involves alterations in cell adhesion and Wnt signaling. Changes in the mRNAs that we observed in the cell adhesion array (Table 1) should be studied at the protein level. For example, as noted above, altered levels of cadherins can affect cell-cell adhesion and also influence Wnt signaling by modulating the availability of β-catenin (59). c-Myc and cyclin D1 are targets of the Wnt pathway in colon carcinoma cells (74).

Another potential mechanism by which MMP-9 influences NSC proliferation focuses attention on the targets of MMP-9 proteolytic activity (68, 70, 71, 75, 76) among which FGFs and their receptors are particularly salient (77–79). In addition, a number of studies suggest close cross-regulation of MMPs and FGF2, for example, studies of the proliferation and migration of smooth muscle cells (80) and mesoangioblast stem cells (81). In endothelial cells, increased MMP-9 led to an increase in bioactive FGF2, which resulted in increased angiogenesis (82). In neuroblastoma cells, FGF2 induced G_1/G_0 cell cycle arrest by up-regulation of p21 (Cip1/Waf1), an inhibitor of G_1/S phase cyclin-dependent kinases, and also increased MMP-9 expression (83). Cyclin D activation occurs in smooth muscle cells induced to proliferate after injury, but this response is greatly reduced in MMP-9 knock-out mice (84). Based on these key correlations in other cell types, future studies in NSCs will focus on an examination of the state of activation of FGF and its receptors as well as the determination of how cell cycle parameters are altered in NSCs in low O_2.

MMP-9 has been shown to be a direct transcriptional target of Wnt signaling in human T-lymphocytes (38), and previous studies have linked Wnt signaling to NSC proliferation and/or differentiation (24, 35, 36, 85). In addition, Wnt signaling was recently linked to HIF-1α stabilization in the response of stem cells to lower O_2 levels (24). In this study, blockade of the canonical Wnt signaling pathway reversed the increase in proliferation and migration in 1% O_2. The increase in MMP-9 expression in NSCs in 1% O_2 was also prevented by blocking Wnt signaling. The present findings indicate that MMP-9 is a target of the Wnt pathway in NSCs and is likely to be regulated at the transcriptional level.

It is notable that several studies have identified genetic variations in hypoxia-inducible transcription factors in human and animal populations exposed to low O_2 environments (86–90). Alterations in other genes correlated with survival in low O_2 environments have been observed in human or animal populations at high altitudes (86–88, 91–93), in *Drosophila* adapted to living at low O_2 levels (94, 95), or in blind mole rats that live in hypoxic underground burrows (89, 90). It is therefore tenable that HIF-1α signaling arose to respond to changing levels of O_2 in the atmosphere over evolutionary time (27, 96, 97) along with the emergence of multicellularity (96, 97). These studies indicate that the regulation of hypoxia-inducible transcription factor stabilization by O_2 homeostasis is an important adaptive mechanism that both cells and organisms utilize to survive and thrive in altered O_2 niches.

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